AMENDMENT

Kindly amend the application, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents, as follows:

IN THE SPECIFICATION

Kindly amend the specification, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents, as follows:

Page 1, between the first and second paragraphs (under the heading "Related Applications") please insert:

--This application is also a continuation-in-part of U.S. application Serial No. 09/169,178 filed October 8, 1998 and issued as U.S. patent No. 6,103,526 on August 15, 2000.--

Page 54, after the first paragraph, and before the three centered stars, please add:

--**EXAMPLE 9 - Human Erythropoietin**

The sequence of human erythropoietin (EPO) is available from GenBank (accession no. X02157). The human EPO gene isolated from a genomic library in bacteriophage Lambda EMBL-3 was used as template to amplify EPO coding sequences by PCR. A construct was made in which EPO's natural signal peptide was replaced by a baculovirus signal peptide. A 5' PCR primer was made that began at the N-terminal residue of the mature peptide. A 3' primer was designed to terminate after the natural stop codon of the EPO open reading frame. After PCR amplification, the resulting EPO gene fragment was inserted into the pMGS12 baculovirus transfer plasmid using standard procedures (Sambrook, J, Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The resulting transfer plasmid contained the coding region from EPO downstream of the polyhedrin promoter, flanked by AcNPV DNA from the EcoRI "I" fragment (Summers and Smith. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, May 1987, Texas A&M University). Confirmation of the correct EPO coding sequence (Jacobs et al. Nature 313 806-10 (1985)) was determined by DNA sequence analysis.

Genomic baculovirus DNA and the transfer plasmids containing the EPO gene were mixed, co-precipitated with calcium chloride, and Sf900+ cells (ATCC CRL-12579, deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2009, under the terms of the Budapest Treaty, under ATCC Designation on Sep. 18,

1998) were transfected as described (Summers and Smith 1987, supra). Recombinant viruses were identified by plaque morphology and several were further plaque purified. Recombinant viruses capable of expressing EPO in infected Sf900+ cellswere identified and used as baculovirus expression vectors to produce recombinant EPO in Sf900+ cells.

Sf900+ cells, at a cell density of 1.5 $x10^6$ cells/ml are infected with the baculovirus expression vector containing the EPO gene at an MOI of 1.0. Sf900+ cells are harvested by centrifugation 72 hours post infection. The cell pellet is discarded and the supernatant containing secreted recombinant EPO ("rEPO") is stored at 4.degree. C. for further processing.

Product purification follows centrifugation, filtration and chromatographic procedures analogous to those presented for influenza virus hemagglutinin (U.S. Pat. No. 5,762,939 and allowed U.S. application Ser. No. 08/453,848, incorporated herein by reference). Thus, EPO can be obtained which is purified to substantial homogeneity or to at least 95% purity. With respect to EPO, DNA encoding EPO and substantial homogeneity of EPO, reference is also made to Lin, U.S. Pat. Nos. 4,703,008, 5,441,868, 5,574,933, 5,618,698, 5,621,080, and 5,756,349. In addition, reference is also made to Wojchowski et al., "Active Human Erythropoietin Expressed in Insect Cells, Using a Baculovirus Vector: A Role For N-Linked Oligosaccharide", Biochimica et Biophysica Acta 910:224-32 (1987), Quelle et al., "High-Level Expression and Purification of a Recombinant Human Erythropoietin Produced Using a Baculovirus Vector", Blood, 74(2):652-57 (1989), Quelle et al., "Phosphorylatable and Epitope-Tagged Human Erythropoietins: Utility and Purification of Native Baculovirus-Derived Forms", Protein Expression and Purification 3:461-69 (1992), and U.S. Pat. Nos. 5,322,837 and 4,677,195. In contrast to any prior EPO from baculovirus expression, EPO in accordance with the present invention can be purified to at least 95% purity or to substantial homogeneity; and, the EPO in accordance with the present invention is produced in relatively high amounts, is glycosylated and secreted, and has physical and biological properties as follows: 25 kD, secreted monomers; stimulates erythropoiesis. stimulates erythropoiesis.

As a particular purification procedure, centrifuged culture supernatant containing rEPO is pH adjusted to pH 8.0 with Tris-base. Proteinatious and non-proteinatious materials bind to precipitating salts, mainly calcium hydroxide, and are removed by centrifugation while rEPO remains in the supernatant. The resulting rEPO containing supernatant is diafiltered into 10 mM Tris-Cl buffer pH 8.0.

The diafiltered rEPO containing supernatant is applied onto DEAE Sepharose and equilibrated with 10 mM Tris-Cl buffer pH 8.0. The rEPO binds weakly and is recovered in the flow-through while contaminants remain bound to the column. Diafiltration into low-conductivity buffer prior to anion-exchange chromatography ensures stronger binding of contaminants and higher degree of purification at this step. The collected DEAE flow-through is diafiltered into 10 mM sodium malonate buffer pH 6.0 and applied to CM Sepharose equilibrated with the 10 mM sodium malonate pH 6.0 buffer. The rEPO binds to CM Sepharose while contaminants flow through the column. The column is then washed with 10 mM sodium malonate buffer pH 6.0 containing 100 mM NaCl, to further remove contaminants. The elute rEPO from the column, a 10 mM sodium malonate buffer pH 6.0 containing 150 mM NaCl is used.

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The eluant containing rEPO is applied to a second CM Sepharose column equilibrated with 10 mM sodium malonate buffer pH 6.0. It is then washed with 10 mM sodium phosphate buffer pH 7.0 and finally, rEPO is eluted in PBS (10 mM sodium phosphate, 150 mM NaCl).

The EPO expressed is glycosylated and has a molecular weight of approximately 25 kD. The amino acid sequence is the same as or analogous to that set forth in literature and patents cited herein. It is quite surprising that the EPO in accordance with the present invention stimulates erythropoiesis as the inventive EPO has glycosylation which does not include sialic acid residues, and there is no O-glycosylation because the EPO is from baculovirus expression; and, any reported recombinant EPO from baculovirus expression heretofore was reported as having no such activity.

In particular, urinary EPO (also known as uEPO) and recombinant EPO produced in mammalian cells are heterogenously glycosylated with complex N- and O-linked oligosaccharides, including sialic acid N-terminal residues, and are acidic proteins, whereas EPO from recombinant baculovirus expression can have a comparably simple saccharide constitution and relative homogeneity, with no sialic acid residues, neutral high-mannose moieties predominating and the highly basic charge density of EPO retained, because of the limited capacity of insect cells to process N-linked oligosaccharides.

Certain literature such as Quelle et al., Blood, supra, at 656, indicates that EPO from expression by insect cells infected with recombinant baculovirus containing DNA coding for EPO is not biologically active due to the lack of sialic acid residues. Further, there is a body of

literature asserting that EPO's "heavy glycosylation" and sialic acid residues are essential for biological activity, see, e.g., Marmont, Tumori 83(4 Suppl 2):S3-15 (1997), Morimoto et al., Glycoconj J 13(6):1013-20 (1996), Higuchi et al., J. Biol. Chem. 267(11):7704-9 (Apr. 15, 1992), Takeuchi et al., Glycobiology 1(4):337-46 (1991), Tsuda et al., Eur. J. Biochem. 188(2):405-11 (1990), Takeuchi et al. J. Biol. Chem. 265(21):12127-30 (1990), Fakuda et al., Blood 73(1):84-9 (1989); Matsumoto et al. Plant Mol. Bio. 27(6):1163-72 (1995) (EPO from tobacco cells lacking sialic acid residues lacked activity).

In contrast, the recombinant EPO of the present invention has an activity of at least 200,000 U/mg (indeed about 500,000 U/mg) and stimulates erythropoiesis. In further contrast to prior EPO, the EPO of the present invention can be isolated using anion exchange and cation exchange chromatography, as opposed to reverse chromatography (used for isolating prior EPO).

Thus, the recombinant EPO of the present invention is distinct from and surprisingly superior to prior EPO.--

IN THE CLAIMS:

Kindly add new claims 96-116, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents, as follows:

--96. (New) A substantially pure, recombinant glycosylated erythropoietin, produced by a baculovirus expression system in cultured insect cells, wherein said erythropoietin has relative homogeneity or is purified to 95% or greater and said erythropoietin stimulates erythropoiesis and has an activity of at least 200,000 U/mg or of about 500,000 U/mg.

97. Erythropoietin of claim 96 wherein said erythropoietin stimulates erythropoiesis and has an activity of at least 200,000 U/mg.

98. Erythropoietin of claim 96 wherein said erythropoietin stimulates erythropoiesis and has an activity of at least 500,000 U/mg.

99. (New) Erythropoietin of claim 96 produced by a method comprising:

culturing insect cells in at least one bioreactor whereby there is an insect cell culture,

wherein the insect cells contain a recombinant baculovirus containing exogenous DNA encoding erythropoietin,

supplying medium in at least one vessel whereby there is culture medium,